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Cellular engineering of ventricular adult rat cardiomyocytes

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Abstract

Objective: Preparation of viable cultured adult cardiomyocytes (vARCs) is a prerequisite for cell-based transplantation and tissue engineering. Ectopic gene expression is important in this context. Here, we present an in vitro cell replating strategy using Accutase™ for cultured vARCs, allowing ectopic gene expression. **Methods:** Cultured vARCs from 6- to 8-week-old rats were used. Transfections with EGFP (enhanced green fluorescent protein) constructs, Mlc-3f-EGFP or α -actinin-EGFP were performed using adenovirus-enhanced transferrin-mediated infection (AVET). Accutase™ (PAA Laboratories, Linz, Austria) was used for the detachment of cultured cells. Immunohistochemical analysis, together with confocal laser microscopy was used for structural analysis of the cells. **Results:** Cultured vARCs could be detached with a high yield (40 to 60%) from primary cultures using Accutase™. The cultivation period plays an important role in the yield of viable cells. Resultant replated vARCs (rep-vARCs) rapidly (1–2 h) acquired a rounded up shape without degradation of their contractile apparatus, which is in contrast to the rod-shaped freshly isolated vARCs (fi-vARCs). The detached cells survived passage through a narrow syringe needle. After seeding, detached cells rapidly attached to various substrates, increased their content of the contractile apparatus, and formed cell–cell contacts within 3 days after reseeded. The detached cells survived passage through a narrow syringe needle. The high recovery of cells after replating enabled the use of the AVET system for gene delivery. AVET is free of infectious particles and does not lead to expression of viral proteins. Transfection of vARCs prior to detachment had a small effect on cell recovery and ectopically synthesized proteins were properly localized after replating. **Conclusions:** Detachment of cultured vARCs using Accutase™ is well compatible with ectopic gene expression and yields a viable transgenic population of vARCs that eventually may be suitable as transgenic cardiomyocyte grafts.

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Keywords: Experimental; Heart; Cellular; Cell culture; Transplantation; Contractile function; Gene therapy; Hypertrophy

1. Introduction

Cardiomyocyte cultures offer the possibility of performing controlled in vitro studies on the pathogenesis of heart muscle. Cell cultures derived from embryonic and neonatal rat heart muscle have frequently been used for such purposes [1]. Obviously, immature and mature cardiomyocytes in culture will differ considerably from one another. The physiology and ultrastructure of isolated adult cardiomyocytes in culture resemble more closely those of cardiomyocytes in the intact heart. Cultures of Ca^{2+} -tolerant ventricular adult rat cardiomyocytes (vARCs) were first described by Jacobson [2] and afterwards refined and

improved [3–7]. In long-term or redifferentiation culture, vARCs first round up after isolation and undergo a transient breakdown of the cytoskeleton, accompanied by cessation of the beating activity. Later the cytoskeleton is regenerated and beating activity is restored [8–13].

Besides their availability for the analysis of basic aspects of morphogenesis, signaling and contractility [14–16], vARC cultures represent an important basis for a possible use as transplants for damaged myocardium. The suitability of vARCs for the repair of myocardial defects has been demonstrated [17]. However, success of graft formation seemed to be correlated to a rounded-up cellular morphology, which usually is only found in vitro for fetal cardiomyocytes [18]. In long-term culture of vARCs

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rounded-up cells may be available after isolation and before spreading. The use of stem-cell derived cells in contrast is hampered by the need to remove undetermined cells prior to injection in order to alleviate the risk of teratoma formation [19].

Genetic engineering leading to a directed modification of the cellular metabolism of vARCs is more easily accomplished *in vitro* than *in vivo* and has been proposed as part of a transplantation strategy. Although adenovirus has been widely used as transgene carrier in such experiments, primarily favored because of its high infection rates, there is nevertheless the disadvantage of a limited persistence of ectopic gene expression [20], and more importantly, of the occurrence of inflammation and fibrosis around the transplantation site. The potential risk arising from the general exposure of the patient to infectious particle has led to the search for alternative systems. The use of the AVET system (adenovirus-enhanced transferrin infection), being free of infectious particles and not involving expression of viral proteins, seemed to be a valuable alternative to be used in conjunction with vARCs. However, so far vARCs, whether untransfected or transfected, could not be released in a viable state after attachment and spreading on the substrate *in vitro*. This is a situation which would make it impossible to perform autologous cell transplantation or tissue repair, or to use cardiomyocytes as host cells for *in situ* gene-based drug delivery.

Here we present strategies using the AVET system in conjunction with the specific protease mixture Accutase™. It allows the *in vitro* generation of tissue-injectable vARCs transfected with desirable DNA constructs, yielding viable adult cardiomyocytes released *in vitro* from the substrate in a rounded-up state. Thus, vARCs can be used for various purposes, among others especially for the transfer of genetically engineered, possibly autologous, heart muscle cells into damaged myocardium.

2. Methods

2.1. Cardiomyocyte isolation and cultivation

Six- to 8-week-old female OFA rats (BRL, Füllinsdorf, Switzerland) were used for the isolation of vARCs. Cells were isolated by retrograde perfusion of the hearts according to an established method [9]. Culture dishes coated with 0.1% gelatin were used throughout. After isolation cardiomyocytes were cultured in a medium containing M199 (Animed, Basel, Switzerland), 10% fetal calf serum (FCS; PAA Laboratories, Linz, Austria), 20 mM creatine monohydrate (Sigma, St. Louis, MO, USA), and 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA). To abolish growth of fibroblasts and other non-cardiomyocyte cell types, 10 μ M cytosine-arabinozide (araC; ICN Biochemicals, Cleveland, OH, USA) was routinely

added to the medium. The investigation has been approved by the Cantonal Committee for Animal Experimentation in Zurich and fully conforms with the 1996 NIH *Guidelines for Care and Use of Laboratory Animals*.

2.2. Accutase™ treatment

If not stated otherwise, primary cultures of vARCs were used after being kept for 9 days in culture. All incubation steps were performed in an incubator at 37 °C in a water saturated atmosphere containing 5% CO₂. To improve the survival of the isolated cells during the replating procedure 5 mM butanedioxy-monoxim (BDM; Sigma) was added to all reagents, except for the preplating medium [21]. To improve detachment, serum and Ca²⁺ were removed from the culture and incubation and washing steps using M199 (10 min) and phosphate-buffered saline (PBS) with EDTA (0.5 mM) were employed for 10 min each. Two to three 15-min digestion steps with addition of fresh Accutase™ (PAA Laboratories) followed. After each digestion cells were collected in ice-cold culture medium to quench the reaction and the cells were then kept on ice for complete termination of the digestion. Detached rounded-up cells were collected by centrifugation for 5 min at 70×g at room temperature. To remove non-cardiomyocytes from the culture, cells were resuspended in culture medium containing 1% FCS and preseeded in uncoated bacterial culture dishes for 1 to 3 h at 37 °C in the incubator for selective attachment of non-cardiomyocytes, e.g., fibroblasts. Subsequently, cells were centrifuged and collected in culture medium, exactly as was done for the primary culture of freshly isolated cardiomyocytes.

2.3. Gene transfer using the AVET system

Transfection of cardiomyocytes with the plasmids pEGFP-N3 (Clontech Laboratories, Palo Alto, CA, USA), α -actinin-EGFP, or pMLC3f-EGFP [21] was performed by using the AVET system, comprising transferrin-poly-(L)lysine conjugates (Tf-pL) with an average chain length of 250 lysine residues, streptavidin-polylysine conjugates (STAV-pL) and biotinylated, psoralen-inactivated adenovirus (AdV) dl1014 [22,23]. Cultured vARCs were generally transfected 6–8 days after the initial isolation procedure. In a typical experiment 3 μ g STAV-pL in 100 μ l HBS [150 mM NaCl, 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), pH 7.3] were mixed with 1.2×10^{10} biotinylated, inactivated AdVdl1014 in 100 μ l HBS and incubated for 30 min at room temperature. Addition of 3 μ g of plasmid DNA in 150 μ l HBS was followed by a 30 min incubation step at room temperature. The mixture was combined with 3 μ g Tf-pL in 150 μ l HBS and incubated for 30 min before the transfection mix was added dropwise to vARCs in 60-mm cultures dishes; the dishes were then centrifuged for 5 min at 100×g to allow rapid aggregation of the transfection

complexes on the cell surface. 4–16 h after the transfection the cultivation medium was replaced.

2.4. Immunohistochemistry

Cells were fixed in 3% paraformaldehyde in PBS, pH 7.4 for 15 min, washed with PBS, and permeabilized with 0.2% Triton X-100 in PBS for 12 min. To minimize non-specific binding, cells were incubated with 0.1% bovine serum albumin in PBS for 20 min. A monoclonal mouse anti-myomesin antibody (clone B4; generated in this laboratory) [24] was used as sarcomeric M-line marker. A polyclonal rabbit anti-cadherin antibody (Sigma–Aldrich) was used to visualize intercalated disc structures. F-actin was stained with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR, USA). The cells were incubated with the primary antibodies for 2–3 h at room temperature, thoroughly rinsed three times with PBS and incubated with secondary antibodies for 1 h. Combinations of fluorescein isothiocyanate (FITC), Cy-3 and Cy-5-conjugated secondary antibodies were used. Cadherin was stained by FITC-conjugated goat-anti-rabbit IgG (Cappel Research, Hamburg, Germany), myomesin was detected by FITC-conjugated goat anti-mouse IgG (Jackson Immuno Research, Hamburg, Germany), or Cy5-

coupled donkey anti-mouse IgG (Jackson Immuno Research). Immunohistochemistry was analyzed using a Zeiss Axiophot fluorescence microscope equipped with Neofluor objectives (Zeiss, Feldbach, Switzerland). Alternatively, a Leica true confocal scanner TCS NT on the inverted microscope Leica DMIRB-E (Leica, Mannheim, Germany) was used.

3. Results

3.1. Detachment of ventricular adult rat cardiomyocytes

Accutase™ treatment allowed detachment and survival of a 9-day spread out primary long-term culture of vARCs from a culture dish containing about 3.0×10^3 cells per 30-mm dish. Fig. 1 shows the time-course of the detachment by employing time-laps video microscopy. Within minutes after Accutase™ treatment the filopodia of the cells became stunted, cell–cell contacts were disrupted, and rounding-up of the cells became evident. Accutase™ treatment allowed the removal of almost all vARCs from the culture dishes, but it was obvious that the in vitro redifferentiation time period of the initial vARC culture critically affected the yield of replated vARCs (rep-

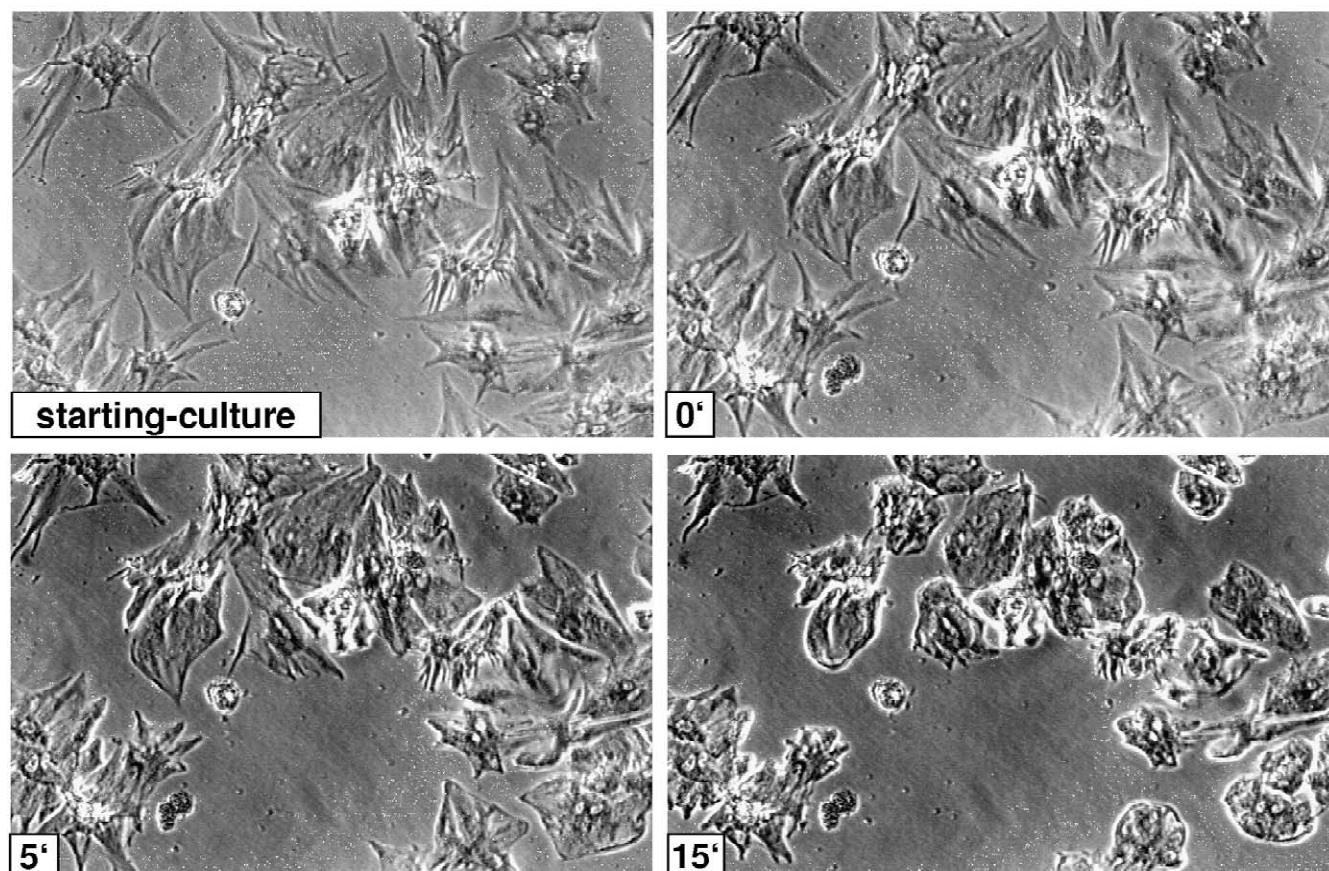


Fig. 1. Live 9-day vARC culture during treatment with Accutase™. Video time-laps records at indicated time points of treatment.

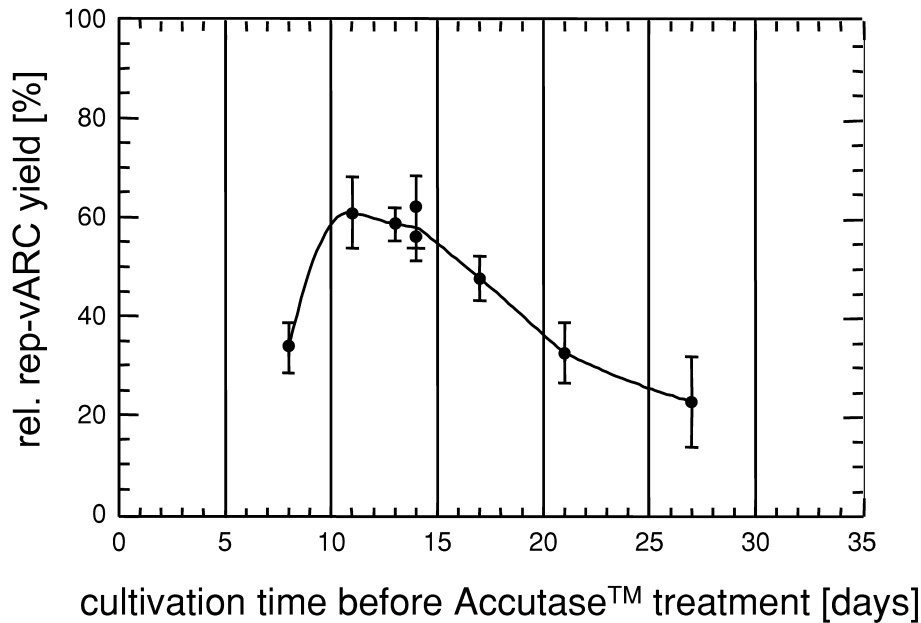


Fig. 2. The yield of rep-vARCs after treatment with Accutase™ is dependent on the pretreatment time (days) in culture. The yield is expressed as ratio of the cell numbers for vARCs on corresponding dishes prior to the treatment, compared to cell numbers for rep-vARCs 3 days after re-plating as determined by immunohistochemical detection of myomesin. Data shown were means \pm S.E.M. of at least three independent experiments.

vARCs) if observed 3 days after re-plating (Fig. 2). A pronounced peak of replating efficiency after Accutase™ was observed between 9 and 15 days after the initial plating of the vARCs. Nine-day cultures of vARCs frequently showed some non-cardiomyocyte cells which, however, clearly were preferably released from the substrate at an early stage after Accutase™ treatment. After separation of the non-cardiomyocyte cells, the remaining fractions represented about 85% of all vARCs recovered.

If rep-vARCs were subjected after 9 days after replating to an additional round of Accutase™ treatment, and a second detachment had taken place, again a good recovery of viable, beating cells could be observed, evidently also providing a means to prolong the life-span of the vARCs in vitro.

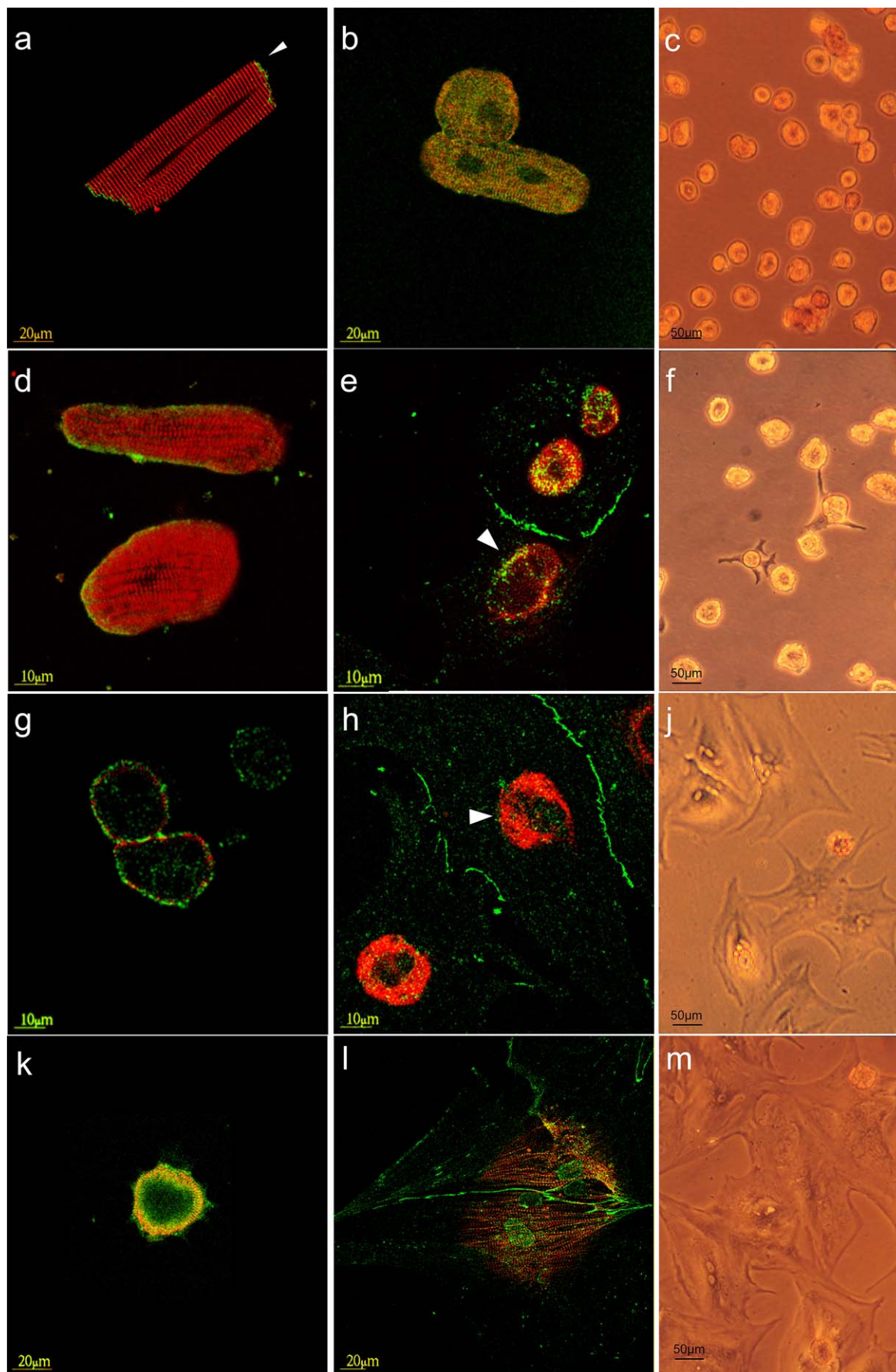
3.2. Cellular behaviour of vARCs after detachment and replating

Whereas freshly isolated vARCs (fi-vARCs) obtained after isolation of cardiomyocytes from adult tissue displayed a rod-shaped morphology with regularly aligned myofibrils, the enzymatic release of polymorphic, spread-out vARCs from the culture dish resulted in a suspension of mainly rounded-up vARCs (Fig. 3a–c). While the

cadherin signal in fi-vARCs was restricted to the former intercalated disc (Fig. 3a, arrow), cadherin was found highly dispersed over the cell surface of the detached vARCs indicating an altered distribution of the protein (Fig. 3b). Beating activity, however, could be observed in a large fraction of the replated cells from detachment throughout the entire observation period definitely being in contrast to fi-vARCs, which resumed beating only after about a week in culture and after spreading on the substrate.

There was a clear time-line difference concerning reformation and spreading of rep-vARCs compared to the initial vARCs, thus pointing to rather altered characteristics of the behavioral pattern. Very briefly after reseeding the Accutase™ treated cells attached to the substrate, spread out and sought contact with neighboring cells. Reformation of new myofibrillar structures started already 1 day after replating (Fig. 3e). This was in contrast to the redifferentiation behaviour of fi-vARCs. At day 1 after replating fi-vARCs were losing the rod-shaped morphology (Fig. 3d) and at day 3 the rounded-up cells had lost most of the sarcomeric structures (Fig. 3g). Whereas vARCs after 5 days in culture just started to spread out and to form myofibrils (Fig. 3k), rep-vARCs spread out right after plating and established new intercalated-disk-like struc-

Fig. 3. Comparison of the redifferentiation process of freshly isolated vARCs (a, d, g, k) and of 9-day-old vARCs after Accutase™ treatment and replating (b, c, e, f, h, j, l, m). Immunohistochemical analysis of cadherin (green) in intercalated disc-like structures, and of myomesin (red) indicating M-lines of myofibrillar sarcomeres. Representative cells are shown directly after isolation from heart tissue (fi-vARCs) (a) or after Accutase™ treatment of a 9-day-old vARC culture (b, c); untreated vARCs at 1 day (d), 3 days (g), 5 days (k) after plating; after Accutase™ treatment at 1 day (e, f), 3 days (h, j), 5 days (l, m) after re-plating. Video time-laps live records (c, f, j, m). Arrows indicate perinuclear localized myofibrils in spread-out cardiomyocytes after Accutase™ treatment. Note different scale bars in k and l.



tures as represented by cadherin expression and localization, thereby allowing new functional interactions and coordinated beating activity already 1 day after replating (Fig. 3e, h, i). Live-shots of rep-vARCs shown in Fig. 3c, f, j, m demonstrate, that already at day 3 after replating spread-out cells closely resembled vARCs after 9 and more days in culture.

The detached rounded-up vARCs survived passage through a syringe needle much better than fi-vARCs. This is a precondition for transplantation of donor cells into infarcted myocardium. It would allow one to transfer vARCs in a state of enhanced and fast redifferentiation competence. As shown in Fig. 4 recovery of fi-vARCs above 2.0 ml/min was significantly diminished, while survival of Accutase™ treated vARCs remained mainly unaffected even at high flow rates.

3.3. Ectopic gene expression in rep-vARC

The AVET system was used to transfect 9-day-old vARCs with vectors encoding EGFP alone (Fig. 5a, c) or as fusion protein with α -actinin (Fig. 5b, d). The morphology and sarcomeric organization of transfected and detached cells were analyzed 5 days after replating. Fig. 5a shows expression of EGFP in one of two neighboring cells. Neither an apparent effect on cell shape nor on the organization of the sarcomers, which were immunostained for the M-band protein myomesin, was observed. Overlay of the red myomesin signal on top of the green intracellular signal of ectopically expressed EGFP resulted in a yellow sarcomeric striation (Fig. 5c). The myofibrillar

distribution of ectopically expressed fusion protein α -actinin-EGFP was analyzed in relation to f-actin stressfibers (Fig. 5d) in spread-out rep-vARCs. Five days after replating; α -actinin was correctly integrated into the Z-line of myofibrillar sarcomers and correlated well with the myofibrillar scaffold formed by f-actin in a more or less perinuclear fashion as had been reported earlier [10]. It was evident that detachment, rounding-up and replating of vARCs did not deteriorate expression and proper localization of fusion proteins in previously transfected vARCs. This was additionally demonstrated by transfection of 9-day-old vARCs with a construct encoding for Mlc-3f-EGFP fusion protein. Recovery of previously transfected cells after replating was not significantly lowered if compared to EGFP alone (Fig. 6)

4. Discussion

Several attempts have been made to generate transplantable cardiac muscle cells to substitute for loss of cardiac function. Recently, stem cells as a source of cardiomyocytes for transplantation became of interest [25], but also several problems like rejection of the transferred cells, the risk of, e.g., teratoma formation arising from the use of undifferentiated, but also ethical issues remain to date unresolved. Evidence, however, is rising, that transplanted cells may have a pronounced potential to locally deliver growth factors [26] and that the use of genetically engineered adult cardiomyocytes for in-situ drug delivery would be appealing. The proper targeting to the site of action followed by electrical and mechanical coupling of the implanted vARCs [27], a high specific metabolic potential [28], and a possible life-time survival in the adult heart, render vARCs particularly suitable host cells. Patient-derived non-dividing adult cardiomyocytes could render such a transplantation approach safe as far as immunological and tumorigenic risks are concerned [29]. In the present study, a strategy for the transfer of genetically engineered adult ventricular cardiomyocytes of rats is shown and data are presented which support the feasibility of such an approach.

Although, in vitro cultures of adult cardiomyocytes have already been performed in this laboratory for quite some time [9,30–32], and a wealth of methods and instrumentation has been established in vitro [15,33], studies of adult cardiomyocytes were limited to cells attached to substrate in rather short-lived primary cultures. Nevertheless, this allowed gene transfer and control of gene expression [34,35] and one could gain insight into various cellular processes in real-time in vitro, e.g., the formation of cell–cell contacts or of sarcomeric structures [15,16] demonstrating the redifferentiation capacity of cultured vARCs. Cell transfer, however, of fi-vARCs into diseased myocard turned out to be problematic, because injection was not easy to perform without losing the cells [36].

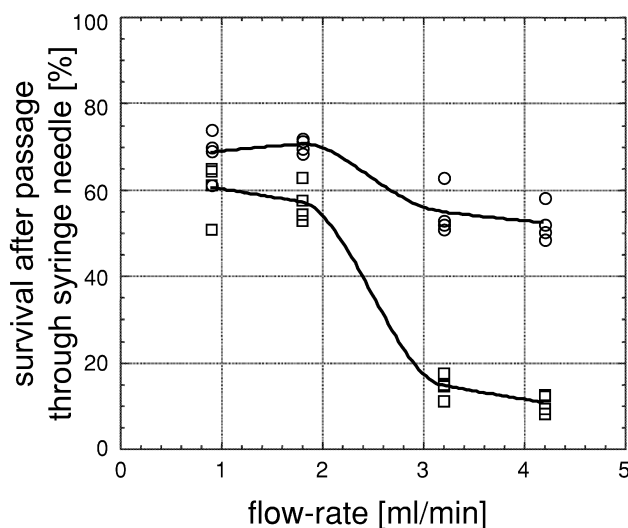


Fig. 4. Nine-day-old vARCs detached by Accutase™ (circles) survive passage through a syringe needle much better than fi-vARCs (squares). Survival is expressed as fraction of surviving fi-vARCs and surviving detached 9-day-old vARCs before and after passage through a 0.2 mm inner diameter needle at volumetric flow-rates indicated. Immunohistochemical detection of myomesin was performed 3 days after plating or replating.

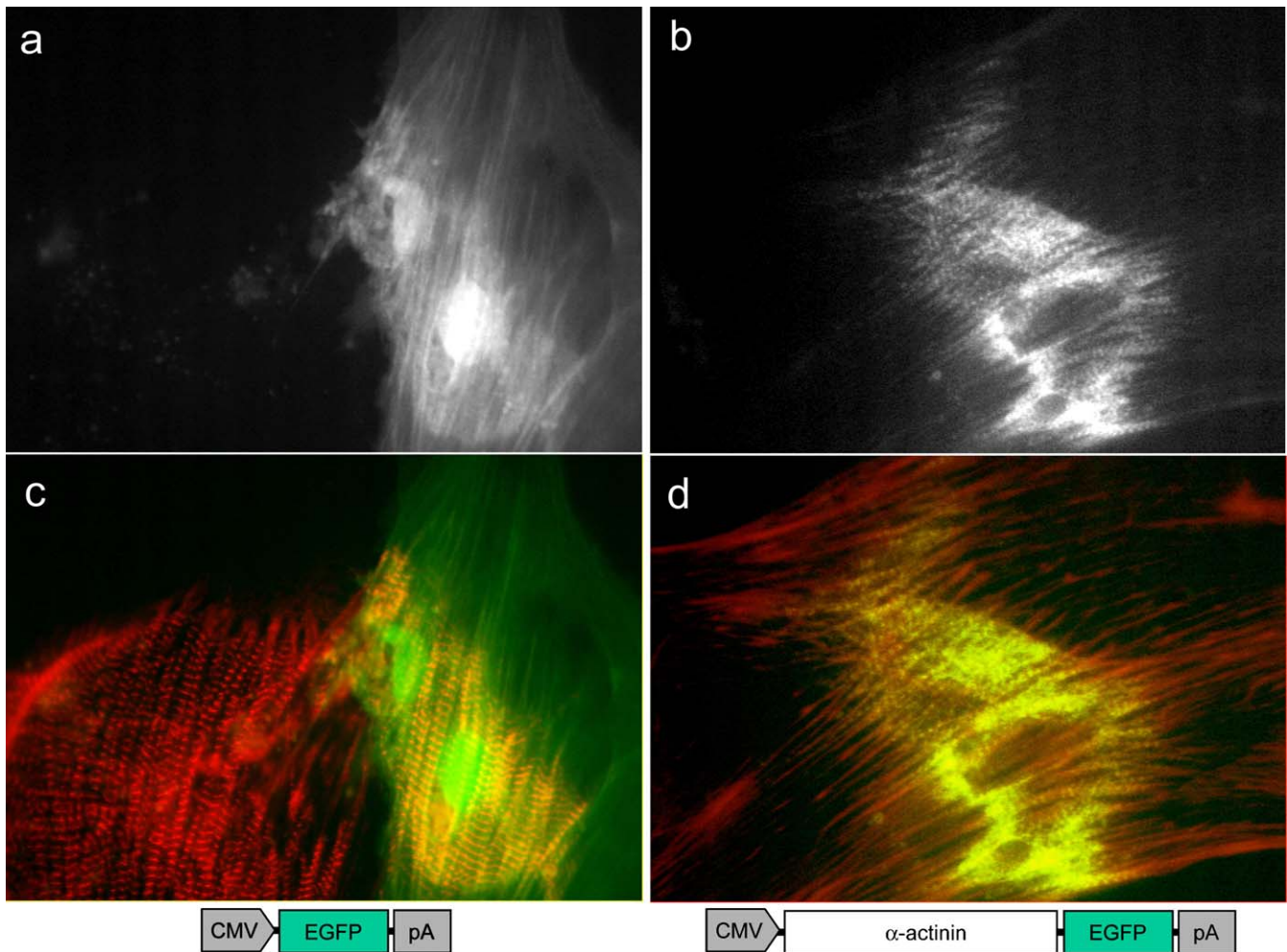


Fig. 5. Transfected vARCs synthesize and correctly target ectopically expressed proteins after Accutase™ treatment and replating. Cells were transfected with expression vectors encoding EGFP (a, c) or an α -actinin-EGFP fusion protein (b, d) 5 days prior to replating. EGFP signals are green in (c, d), myomesin is red in (c) and f-actin is red in (d). Superposition green/red is yellow. Corresponding expression cassettes are shown.

Neonatal cardiomyocytes had been successfully injected into cardiac tissue, indicating the feasibility of using cardiomyocytes for this purpose [37], but show on the other hand only a minor differentiation capacity. The here reported treatment of cultured adult rat cardiomyocytes, vARCs, with Accutase™ led to the formation of viable, rounded-up cells, what was quite in contrast to prior attempts to detach viable adult cultured cardiomyocytes by the use of trypsin or other reagents.

Thus, several goals could be achieved. Rounded-up, highly differentiated adult cardiomyocytes allowing the passage through narrow needles required for injection, could be produced. We have also found that the detached and replated transgenic adult cardiomyocytes show rapid spreading and a high, 40 to 60%, recovery in viable cells. Multiple rounds of detachment by Accutase™ resulted in a similar recovery of viable cells thus providing an additional means to prolong the life-span of vARCs in culture.

The observed rapid formation of new cell–cell contacts and the extended formation of myofibrils might be an

important advantage for cell transplantation and for formation of grafts. Consequently, a rapid functional integration of transplanted cells into existing cardiac structures and a high yield of surviving cells at the injection site could be expected. Indeed, as had been reported earlier the suitability of neonatal and fetal cardiomyocytes is correlated with this characteristic [18]. Transplantation of genetically engineered vARCs will much depend on the availability of viable detached cardiomyocytes. Therefore, it was important that we could show that also transfected vARCs could be detached by Accutase™ and viably replated without loss of the transfected transgenes. Expression of ectopic proteins was not impaired by the detachment. Transfection of EGFP alone was somewhat better than transfection of the Mlc-3f-EGFP fusion protein, which is an observation being made for other cell types as well. However, during replating of Mlc-3f-EGFP transfected cells and untransfected cells were equally well transferred. In the context of a possible gene therapy the above mentioned characteristics are important.

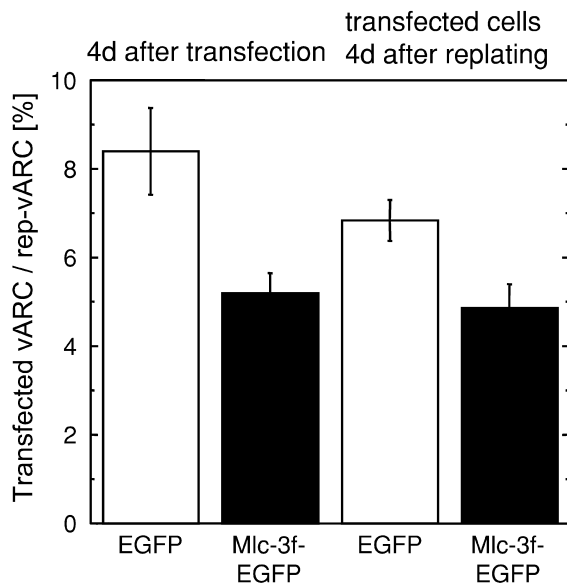


Fig. 6. The nature of the ectopically expressed gene has only a negligible effect on the recovery of transfected cells after replating. vARCs were transfected after 9 days in culture with expression vectors encoding EGFP (open columns), or Mlc-3f-EGFP fusion protein (solid columns). Four days after transfection, cultures were either fixed (left pair), or fixed 4 days after Accutase™ treatment (right pair). The fraction of transfected cardiomyocytes was assessed using the EGFP-signal of the ectopically expressed proteins together with the immunohistochemical signal of myomesin. Data shown were means \pm S.E.M. of four independent determinations.

In this study we used a virtually non-viral system for gene-transfer, no active viral particles were involved, no viral genes expressed, thus abolishing several severe drawbacks of adenoviral and adenovirus derived strategies, thereby demonstrating a promising alternative route towards genetic engineering of cardiomyocytes for in vivo applications. The presented strategy and results have led to the formation of a versatile genetically engineered population of adult cardiomyocytes. They will represent an in vitro model for the structural, electrophysiological, and biochemical analysis of transgenic effects appearing in vivo and may as well allow the formation of transgenic cardiac autografts for the restoration of impaired cardiac-function as had been proposed earlier [26].

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